

MELANIN-INDEPENDENT ACCUMULATION OF TURGOR PRESSURE IN
APPRESSORIA OF *PHAKOPSORA PACHYRHIZI*

BY

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THESIS

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ABSTRACT

Appressoria of some plant pathogenic fungi accumulate turgor pressure that produces a mechanical force enabling the direct penetration of hyphae through the epidermis. Melanin functions as an impermeable barrier to osmolytes, which allows appressoria to accumulate high turgor pressure. Deficiency of melanin in appressoria reduces turgor pressure and compromises the infection process. In *Phakopsora pachyrhizi*, the soybean rust pathogen, the appressoria are hyaline. Our objective was to ensure the absence of a melanin layer specifically between the appressorial cell wall and plasma membrane, as well as to determine the turgor pressure of *P. pachyrhizi* appressoria. We demonstrated that two melanin biosynthesis inhibitors neither reduced turgor pressure nor compromised the infection process. Transmission electron microscopy also showed the absence of a melanin layer between the appressorial cell wall and plasma membrane. In addition, the turgor pressure of *P. pachyrhizi* appressoria ranged from 5 to 6 MPa based on extracellular osmolytes used to simulate different osmotic pressures. This is the first report showing that turgor pressure accumulation of *P. pachyrhizi* appressoria was independent of melanin.

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INTRODUCTION

Soybean rust, caused by the fungus *Phakopsora pachyrhizi* Syd., is an economically important disease of soybean that results in significant yield losses (24, 28). The infection and colonization of *P. pachyrhizi* in soybean leaves is well documented (10, 18, 22, 37) and reviewed (13, 14). Briefly, after a urediniospore is deposited on a leaf, it germinates to form a germ tube that results in a terminal appressorium. The infection continues by the formation of an appressorial cone inside the appressorium where the penetration hyphae breaks through the plant epidermis, and primary hyphae grow to invade the mesophyll tissue. The hyphae further differentiate into a haustorial mother cell and develop haustoria inside the mesophyll cells. The observation through the transmission electron microscopy not only elucidated the infection process, but also demonstrated that the leaf penetration was dependent on both mechanical force from appressoria and plant cell wall degradation (10).

In many plant pathogenic fungi, penetration of the plant epidermis occurs by mechanical force as a result of melanized appressoria accumulating high turgor pressure (2, 23). Several types of melanin, including dihydroxynaphthalene (DHN) melanin and L-3,4- dihydroxyphenylalanine (DOPA) melanin are implicated (5). The infection of rice by *Magnaporthe oryzae* demonstrated the importance of melanized appressoria by treating the fungus with tricyclazole, that inhibits DHN melanin biosynthesis, or by using albino appressorial mutant strains, both of which resulted in limited infection of rice (7, 15, 17). It was shown that *M. oryzae* accumulated glycerol as an osmolyte inside appressoria that allowed inflow of surrounding water (8, 16) and the melanin layer prevented the outflow of osmolytes, resulting in high turgor pressure inside appressoria (27). A similar study

has not been done with *P. pachyrhizi* and it is not known if turgor pressure accumulation in appressoria is dependent on melanin or not. Although the appressoria *P. pachyrhizi* appear to be hyaline, the absence of a melanin layer specifically between the appressorial cell wall and plasma membrane maybe present and may provide appressoria turgor pressure similar to what was demonstrated in *M. oryzae* (7, 15, 17).

Several methods have been developed to measure turgor pressure of appressoria (2). Among these, extracellular osmolytes facilitate the observation of plasmolysis or cytorrhysis of appressoria. For example, appressoria treated with different concentrations of polyethylene glycol (PEG) showed that 8 MPa caused cytorrhysis in 50% of *M. oryzae* appressoria (16). The same approach also was applied to other plant pathogenic fungi, including *Colletotrichum* species that also were shown to rely on melanized appressoria for successful infection (23). For *C. kahawae*, the causal agent of coffee berry anthracnose, the DHN-melanin synthesis in appressoria allowed for the accumulation of 2.6 MPa turgor pressure, which was shown to be necessary for infection of both leaves and fruits (6). For *C. graminicola*, which causes anthracnose of maize and other grains, the turgor pressure of appressorium was estimated at 5.4 MPa (3). For *Erysiphe graminis* f. sp. *hordei*, the powdery mildew pathogen of barley, a turgor pressure of 2.6 MPa was needed with the assistance of cell wall degrading enzyme for penetration and infection (30). Appressorial turgor pressures in plant pathogens have been shown to range from 0.3 to 8 MPa (Table 1).

Previous reports indicate that increased turgor pressure may be dependent on melanin accumulation in appressoria. The observation of hyaline appressoria of *P. pachyrhizi* raised questions of whether melanin was absent in the appressorial cell wall or

if a thin melanin layer existed in a way that was invisible to light microscopy, and whether appressoria accumulated turgor pressure as reported in other plant fungal pathogens. In this study, the melanized appressoria of soybean anthracnose pathogen, *C. truncatum*, were used as a comparison to show the differences of turgor pressure accumulation, morphology, and pathogenicity in response to melanin inhibitors. The objective of this study was to determine if appressoria of *P. pachyrhizi* accumulated turgor pressure and to determine if the turgor pressure accumulation was independent of melanin synthesis in between the appressorial cell wall and the cytoplasmic membrane.

MATERIALS AND METHODS

Fungal and plant materials. The susceptible soybean (*Glycine max*) cultivar Williams 82 was grown at 25°C in a growth chamber set for 12 h light (380 mmol m⁻² s⁻¹) per day. The obligate soybean rust pathogen, *P. pachyrhizi* isolate FL 07-1, was maintained on the first trifoliolate detached soybean leaflets. To prepare leaves for inoculation, leaflets were detached from soybean plants and rinsed in 0.1% Tween 20 (Fisher Scientific, MA, USA). Leaflets were placed abaxial side up in clamshell boxes on top of moistened paper towels. Leaflets were inoculated from a previously prepared spore solution (0.65×10^5 spores per ml) until the leaflet was fully covered as the drops dispersed on the Tween 20 rinsed leaflets. The inoculated leaflets were incubated in the dark at $23 \pm 2^\circ\text{C}$ for 12 h and then at 20°C with 16 h light (380 mmol m⁻² s⁻¹) per day. Soybean anthracnose pathogen, *C. truncatum* isolate IL28C, was maintained on potato dextrose agar (PDA) in a growth chamber set at 25°C with 12 h light.

The effect of melanin inhibitors on spore germination, appressorial formation and pathogenicity. The urediniospores of *P. pachyrhizi* and conidia of *C. truncatum* were collected from infected detached soybean leaflets and a culture grown on PDA, respectively. Spores were suspended in 0.1% Tween 20 solution, and adjusted to 0.5×10^5 spores per ml. Tricyclazole (Sigma-Aldrich, MO, USA) and niacin (Sigma-Aldrich), known melanin inhibitors (15, 20), were prepared as 500 µM stocks and stored at 4 °C. Tricyclazole was first dissolved in 0.01% ethanol. Each inhibitor was added to each spore suspension in 0.1% Tween solution and adjusted to testing concentrations. A 30 µl spore suspension was spotted on cover glass (No.1 Corning, NY, USA) and incubated at $23 \pm 2^\circ\text{C}$ in the dark. At 6, 12, 24, and 48 hr post inoculation (hpi) for *P. pachyrhizi* and 16,

24, 48 hpi for *C. truncatum*, spore germination and appressoria formation (with or without melanin inhibitors) were measured under the Olympus compound microscope BX51. This was conducted three times using 50 spores for each of the three biological replications totaling 450 spore observations for each fungus at each time point.

To determine the effect of the melanin inhibitors on pathogenicity of the two fungi, spores collected as previously described were suspended in 50 mM niacin, 50 mM tricyclazole, and 0.1% Tween 20 at a concentration of 0.65×10^5 and 3×10^6 spores per ml for *P. pachyrhizi* and *C. truncatum*, respectively. The detached leaflets were uniformly sprayed with each spore suspension and incubated at $23 \pm 2^\circ\text{C}$ in the dark for 12 and 36 hr for *P. pachyrhizi* and *C. truncatum*, respectively. The leaflets were arranged in moist chambers in a completely random design with three leaflets per treatment. The leaflets were incubated for 10 days at 20°C with 16 h light for *P. pachyrhizi* and at 25°C with 12 h light for *C. truncatum*. To quantify *P. pachyrhizi* uredinia, two 5 mm leaf disks were randomly sampled per leaflet from three randomly selected leaflets. The leaf disks were bleached in a mixture of absolute ethanol-acetic acid (3:1 vol-vol) for 12 h to remove pigments (4, 37). The leaf disks were then floated in lactophenol (Fisher Scientific, NJ, USA) for 6 h and stained with 0.1% cotton blue (ICN Pharmaceuticals Inc., NJ, USA) in lactophenol for 12 h. The leaf disks were washed with distilled water twice and stained uredinia were examined visually under the Olympus stereoscope SZX16. Three biological replications were conducted.

Turgor pressure measurements and permeability differences between the cell walls of urediniospores and appressoria. Polyethylene glycol (PEG) 8000 (Sigma-Aldrich) solutions were used to conduct plasmolysis tests. The conversion of PEG

concentration to osmotic pressure Π (MPa) is given by the equation: $\Pi = \alpha C + \beta C^2$, while Π is the osmotic pressure (MPa), C is the molar concentration (M) of PEG and α and β are constants from different molecular weights of PEGs (26). The α and β constant were interpolated based on the regression equation for alpha as $y_{\alpha} = (-2 \times 10^{-7})x^2 - 0.0021x + 1.4356$, $R^2 = 0.9487$, and beta as $y_{\beta} = (4 \times 10^{-5})x^2 - 0.0547x + 41.444$, $R^2 = 0.9969$, with the y as the speculated constant from the regression equation and x the molecular weight of PEGs (16). Based on the analysis, the α and β constant for PEG 8000 was -28.16 and 2163.84 , respectively. Hence, the solutions in different osmotic pressures were prepared based on the transfer equation: $\Pi = -28.16 C + 2163.84 C^2$. A 3.22 molar glycerol (Fisher Scientific) was prepared to generate the 5.8 MPa osmotic pressure as previously reported (8).

For the turgor pressure measurements, the water drop on the cover glass was removed with a Kimwipe (Kimberly-Clark, Knoxville, TN) and replaced with 30 μ l of PEG 8000 solution at 6, 12, 24, and 48 hpi for *P. pachyrhizi* and 16, 24, 48 hpi for *C. truncatum*. After 5 minutes of incubation with PEG 8000 solution, the number of appressoria with plasmolysis was counted. For each of the osmotic PEG 8000 solution treatments, 100 spores were counted at each time point with three biological replications. Linear regression analysis was used to determine the concentration that caused 50% plasmolysis of the appressoria and was used as the turgor pressure of the appressoria as previously described in other fungal appressoria (6, 16).

To quantify the permeability of the cell wall of urediniospores, a 5.8 MPa glycerol solution together with a 6 MPa PEG8000 solution as applied to appressoria 12 hpi for 5

minutes. In this experiment there were 100 spores counted within each of three biological replications.

Data analyses. The data collected from replicated experiments were subjected to ANOVA using R 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria). If the linear model was significant, Tukey's HSD test (R 3.0.1) was used to separate means. The variables analyzed included the number of uredinia stained in plant tissues to determine the effect of melanin inhibitors on pathogenicity, turgor pressure measurements of appressoria in the experiments that used tricyclazole and niacin, and the percentage of plasmolysis and cytorrhysis that resulted when urediniospores were treated with PEG and glycerol.

Transmission electron microscopy (TEM) observations. Soybean leaflets containing 12 hpi appressoria were embedded in 2% agar. The leaf tissues were fixed in a Karnovsky's Fixative in phosphate buffered 2% glutaraldehyde and 2.5% paraformaldehyde. The tissue was washed in Sorenson's phosphate buffer at pH 7.2 with no further additives, and infiltrated by 2% osmium tetroxide for 2 hr followed by the addition of 3% potassium ferrocyanide for 30 minutes. After rinsing with water, saturated uranyl acetate was added for en bloc staining. A vacuum was used to facilitate the efficiency of infiltrations using a vacuum jar connected to a pump (Air Cedet Portable Vacuum/Pressure Station, Barnant Thermo Scientific, Barrington, IL)

The tissue was dehydrated in a series of increasing concentrations of ethanol. Acetonitrile was used as the transition fluid between ethanol and the epoxy. An infiltration series was done with an epoxy mixture using the epon substitute Lx112. The resulting blocks were polymerized at 80°C for 12 h, trimmed, and sectioned with diamond knives. Sections

were stained with filtered uranyl acetate and Luft's instant lead citrate (Ladd Research Inc., Williston, VT) following previously described procedures (12, 25), and then examined and photographed with a Hitachi H600 Transmission Electron Microscope.

RESULTS

The effect of melanin inhibitors on spore germination, appressorial formation and pathogenicity. To avoid observation bias under the light microscope and to support the independent of melanin synthesis during appressoria formation of *P. pachyrhizi*, we applied DHN and DOPA melanin inhibitors, tricyclazole and niacin, respectively, to ensure the absence of melanin in *P. pachyrhizi* appressoria. There was no effect of the melanin inhibitors on spore germination and appressorial formation of *P. pachyrhizi* and *C. truncatum* spores. The appressoria of *P. pachyrhizi* were hyaline with no pigmentation on the appressorial cell wall. In contrast, the appressoria of *C. truncatum* were opaque and melanized. Appressoria of *P. pachyrhizi* treated with tricyclazole and niacin were visually identical to the appressoria in the 0.1 % Tween 20 solution control. Appressoria of *C. truncatum* treated with 50 mM tricyclazole blocked melanin synthesis and turned the intermediate product into reddish compounds (7, 11, 36), while niacin did not eliminate the melanin indicating the opaque color was DHN-melanin (Fig. 1).

Soybean leaflets inoculated with conidia of *C. truncatum* mixed with tricyclazole produced fewer necrotic spots than conidia mixed in niacin or in the Tween 20 control (Fig. 2A). Soybean leaflets, inoculated with urediniospores of *P. pachyrhizi* suspended in tricyclazole or niacin, were visually indistinguishable from the control based on the appearance of sporulating uredinia (Fig. 2B). Based on counts of stained uredinia (Fig. 2C), there were no significant differences between the treatments (Fig. 2D).

Turgor pressure measurements and permeability differences between the cell walls of urediniospores and appressoria. A fully mature appressorium of *P. pachyrhizi* was defined as as one in which all the cytoplasm had migrated and was delimited from

the germ tube into the appressorium. The highest appressorium turgor pressure was 6.01 ± 0.93 MPa at 12 hpi. The accumulation peaked at 12 hpi and decreased till 48 hpi. Neither tricyclazole nor niacin affected the accumulation of turgor pressure (Table 2). When the melanin inhibitors were used at different concentrations (50 mM, 150 mM and 250 mM), there was no significant reduction in turgor pressure of appressoria of *P. pachyrhizi* (Table 3).

A fully mature appressorium of *C. truncatum* was defined as an appressorium completely melanized, or about the same size of the swollen tip of the germination tube when tricyclazole was applied. The highest appressorium turgor pressure was 4.58 ± 0.25 MPa at 24 hpi. Niacin did not alter turgor pressure accumulation; however, tricyclazole significantly ($P < 0.05$) reduced the pressure at 16 hpi (3.56 ± 0.29 MPa) and 24 hpi (2.79 ± 0.15 MPa) (Table 2).

Based on the treatments of 6 MPa PEG 8000 and 5.8 MPa glycerol solutions, plasmolysis of *P. pachyrhizi* appressoria was observed. In both treatments, the cell wall remained at the original position while the cytosol shrank inward and left a space inside the appressorium (Fig. 3). The empty urediniospores responded to PEG 8000 and glycerol differently. Only $10.67 \pm 2.52\%$ of empty urediniospores had cytorrhysis, while the majority remained unchanged under glycerol osmotic pressure. However, the $73.67 \pm 10.02\%$ of empty urediniospores exhibited cytorrhysis under the osmotic pressure generated by the PEG 8000 solution, which showed a significantly different response to the molecular size ($P < 0.05$). These observations suggested that both the large molecule PEG 8000 and the small molecule glycerol were permeable through the non-melanized

cell wall of *P. pachyrhizi* appressoria, but the permeability was different in the case of the empty urediniospores.

TEM observations. The appressoria had lost their spherical shape upon penetration. In response to infection, soybean cells would form papillae to counteract the infection at the penetration site (Fig. 4A). When penetration began, an appressorial cone was observed (Fig. 4A, C-E). The penetration hyphae invaded through the epidermis and developed into primary hyphae, which colonized the leaf tissue (Fig. 4E). However, no melanin layer was observed in the cell walls of appressoria in all these stages, but a grey waxy layer was found locally (Fig. 4B-D). Although dark material was associated with the appressorial cone near the penetration site, the accumulation of this dark material did not follow the appressorial cell wall (Fig. 4B, D-F) indicating the absence of a melanin layer between the cytosol and appressorial cell wall of *P. pachyrhizi*.

DISCUSSION

The accumulation of turgor pressure in appressoria of *P. pachyrhizi* was independent of melanin. The DHN and DOPA melanin inhibitors, tricyclazole and niacin, did not alter the development or morphology of appressoria nor was there any interruption of the infection process. In comparison, the turgor pressure of *C. truncatum* declined significantly from 4.58 MPa to 2.79 MPa and the melanin in appressoria disappeared in the presence of the DHN-melanin inhibitor tricyclazole. When treated with hyperosmotic PEG 8000 solution, most of the appressoria of *P. pachyrhizi* plasmolyzed with appressorial cell wall remaining in position and the detached cytosol shrinking inward. When treated with hyperosmotic glycerol, nearly all of the appressoria plasmolyzed similar to the PEG 8000 treatment. However, the empty urediniospores responded differently; the PEG solution caused cytorrhysis in the empty urediniospores and plasmolysis of the appressoria while glycerol caused plasmolysis in both empty urediniospores and appressoria. This indicated that the appressorial cell walls of *P. pachyrhizi* were permeable to small molecules to large polymers such as PEG 8000; however, the cell walls of urediniospores were only permeable to small molecules like glycerol, reported to have ability to diffuse through the non-melanized appressorial cell wall in *M. oryzae* (8).

We did not observe a melanin layer in the TEM micrographs at any of the stages of appressoria formation and penetration. We observed that spherical appressoria collapsed or deformed upon penetration, consistent with a previous report (18), and similar to a report on a closely related rust fungus, *P. apoda*, where appressoria were observed as wrinkled or polymorphic rather than spherical at the penetration stage (1). The observed

penetration process was consistent to previous reports (10, 18), but we noticed an unidentified grey layer or substance existing between the appressorial cell wall and cytosol. The layer is different from dark melanin, and likely a waxy layer or a polysaccharide layer that serves to join the appressorial cell wall and plasma membrane. The waxy layer may make a minor contribution on preventing the outflow of cytosol substances because the appressorial cell wall was permeable to molecules as large as PEG8000 in our tests.

Fungi produce different kinds of melanin that function primarily to protect fungal structures and aid in survival. Based on the composition of their monomer, they are either DOPA-melanin or DHN-melanin, and both enhance cell tolerance against radiation, extreme redox, temperature conditions, and degradation of enzymes (5). In addition, melanized appressoria allow the accumulation of high turgor pressure, which generates the mechanical force to penetrate plant cell walls (9, 17, 23).

The identification of the melanin inhibitor, tricyclazole, and the melanin-deficient mutants in the rice blast fungus, *M. oryzae*, led to the discovery that melanin was essential for pathogenicity, and launched studies that focused on the function of melanin in appressoria (7, 15). The location of DHN melanin between the plasma membrane and the appressorial cell wall was first characterized by TEM, and further supported by discovering that the melanin layer disappeared with treatment of tricyclazole. The melanin layer was impermeable to molecules larger than water, based on high concentrations of KCl solution treatments, which allowed the appressoria to maintain osmolytes inside and recruit water to maintain turgor pressure (15). The first report of measuring the appressorial turgor pressure of *M. oryzae* used extracellular PEG solutions

differing in osmolality to estimate the osmotic pressure that equates to 50% cytorrhysis and found that the turgor pressure in the appressoria was up to 8 MPa (16). The osmolytes in the appressoria of *M. oryzae* were characterized as mainly glycerol, which were shown to leak out of appressoria in melanin-deficient mutants (8). Cytorrhysis of appressoria occurred when hyperosmotic glycerol was applied for both wild-types and melanin-deficient mutants; however, the melanin-deficient appressoria recovered through plasmolysis by the equilibrium of extracellular hyperosmotic glycerol (8). With this evidence, the authors concluded that glycerol was capable of diffusing between the melanin-deficient appressorial cell walls, but not the melanized appressorial cell walls. Therefore, the function of the melanin layer was shown to prevent the leakage of osmolytes like glycerol through appressorial cell walls (8, 27). An alternative function for melanin in appressoria recently suggested that instead of a build-up of impermeability, melanin prevents the deleterious damage of cell wall degrading enzymes produced by the fungus, and it was shown that melanized and non-melanized appressoria of *C. graminicola* did not differ from the concentration of osmolytes based on measurements made by using a Mach-Zehnder-Microscopy; however, the non-melanized appressoria were more sensitive to extracellular lysing enzymes (21). This suggested that the outflow of osmolytes through the plasma membrane could be limited (21). If this is the case, and the lipid bilayer is indeed sufficient for maintaining high osmolytes in the cytosol, the measurements of lower glycerol in melanin-deficient strains of *M. oryzae* in comparison to the wild type may not be plausible as was previously reported (8). Since melanin does not appear not to be required for turgor pressure accumulation of *P. pachyrhizi* and in the

melanin deficient mutants of *C. graminicola* (21), it is not clear how appressoria maintain osmolytes and generate high turgor pressure.

In *M. oryzae*, DHN-melanin was shown to be responsible for the dark appressoria. The pathogenicity was reduced in mutants deficient in the DHN-melanin synthesis pathway (7). In the DHN-melanin synthesis pathway, polyketide synthase (PKS) polymerizes the precursor acetate that terminates in a 1,8-DHN as the monomer for the melanin polymer. For the albino mutants (pks-) of *M. oryzae*, *C. lagenarium*, and *C. graminicola*, they were not able to produce melanin (7, 21, 32). Several EST and RNA-Seq studies completed during urediniospore germination and appressoria or uredinia formation reported no PKS genes in *P. pachyrhizi* (29, 31, 34, 35). Although the sensitivity on detecting PKS genes by EST and RNA-Seq methods depend on the coverage and depth of sample libraries, the absence of PKS genes detection during urediniospore germination and appressoria formation may suggest that the expression of PKS genes is not necessary for *P. pachyrhizi* at these stages. Furthermore, a genome-wide study also indicated the orthologs of PKS genes rarely exist in fungi belonging to the *Pucciniomycotina* including *Melampsora laricis-populina*, *Mixia osmundae* IAM 14324, *Puccinia graminis*, *Rhodotorula graminis* strain WP1 and *Sporobolomyces roseus* (19). As one of the members in *Pucciniomycotina*, it seems likely that *P. pachyrhizi* has no PKS genes. The whole genome sequencing will provide advanced details on the genome structure of *P. pachyrhizi*.

This appears to be the first case of melanin-independent turgor pressure accumulation in *P. pachyrhizi* appressoria. Melanin inhibitors failed to affect the appressorial morphology, development, and turgor pressure, or the pathogenicity of *P.*

pachyrhizi. No melanin layer was observed in between the cytosols and appressorial cell walls based on TEM micrographs, even though the appressorial turgor pressure of *P. pachyrhizi* was measured at 5 to 6 MPa. Our study provides evidence to support that melanin may not be needed to build-up an impermeable barrier, as turgor pressure accumulation was independent of melanin. It remains unclear how *P. pachyrhizi* manages the osmolytes and turgor pressure inside appressoria without creating an impermeable barrier.

LITERATURE CITED

1. Adendorff, A., and Rijkemberg, F. H. J. 2000. Scanning electron microscopy of direct host leaf penetration by urediospore-derived infection structures of *Phakopsora apoda*. Mycol. Res. 104:217-324.
2. Bastmeyer, M., Deising, H. B., and Bechinger, C. 2002. Force exertion in fungal infection. Annu. Rev. Bioph. Biom. 31:321-341.
3. Bechinger, C., Giebel, K.-F., Schnell, M., Leiderer, P., Deising, H. B., and Bastmeyer, M. 1999. Optical measurements of invasive forces exerted by appressoria of a plant pathogenic fungus. Science 285:1896-1899.
4. Bonde, M. R., Nester, S. E., Austin, C. N., Stone, C. L., Frederick, R. D., Hartman, G. L., and Miles, M. R. 2006. Evaluation of virulence of *Phakopsora pachyrhizi* and *P. meibomia* isolates. Plant Dis. 90:708-716.
5. Butler, M. J., and Day, A. W. 1998. Fungal melanins: A review. Can. J. Microbiol. 44:1115-1136.
6. Chen, Z., Nunes, M. A., Silva, M. C., and Jr., C. J. R. 2004. Appressorium turgor pressure of *Colletotrichum kahawae* might have a role in coffee cuticle penetration. Mycologia 96:1199-1208.
7. Chumley, F. G., and Valent, B. 1990. Genetic analysis of melanin-deficient, nonpathogenic mutants of *Magnaporthe grisea*. Mol. Plant-Microbe Interact. 3:135-143.
8. de Jong, J. C., McCormack, B. J., Smirnov, N., and Talbot, N. J. 1997. Glycerol generates turgor in rice blast. Nature 389:244-245.

9. Deising, H. B., Werner, S., and Wernitz, M. 2000. The role of fungal appressoria in plant infection. *Microbes Infect.* 2:1631-1641.
10. Edwards, H. H., and Bonde, M. R. 2011. Penetration and establishment of *Phakopsora pachyrhizi* in soybean leaves as observed by transmission electron microscopy. *Phytopathology* 101:894-900.
11. Engh, I., Nowrousian, M., and Kuck, U. 2007. Regulation of melanin biosynthesis via the dihydroxynaphthalene pathway is dependent on sexual development in the ascomycete *Sordaria macrospora*. *FEMS Microbial Lett.* 275:62-70.
12. Glauert, A. 1980. Practical Methods in Electron Microscopy. Part 1: Fixation, Dehydration and Embedding of Biological Specimens. Elsevier North-Holland Inc.
13. Goellner, K., Loehrer, M., Langenbach, C., Conrath, U., Koch, E., and Schaffrath, U. 2010. *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust. *Mol. Plant Pathol.* 11:169-177.
14. Hartman, G. L., Hill, C. B., Twizeyimana, M., Miles, M. R., and Bandyopadhyay, R. 2011. Interaction of soybean and *Phakopsora pachyrhizi*, the cause of soybean rust. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* 6:doi: 10.1079/PAVSNNR20116025.
15. Howard, R. J., and Ferrari, M. A. 1989. Role of melanin in appressorium function. *Exp. Mycol.* 13:403-418.
16. Howard, R. J., Ferrari, M. A., Roach, D. H., and Money, N. P. 1991. Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proc. Natl. Acad. Sci. USA* 88:11281-11284.

17. Howard, R. J., and Valent, B. 1996. Breaking and entering: Host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. Annu. Rev. Microbiol. 50:491-512.
18. Koch, E., Ebrahim Nesbat, F., and Hoppe, H. H. 1983. Light and electron microscopic studies on the development of soybean rust (*Phakospora pachyrhizi* Syd.) in susceptible soybean leaves. Phytopathology Z. 106:302-320.
19. Lackner, G., Misiek, M., Braesel, J., and Hoffmeister, D. 2012. Genome mining reveals the evolutionary origin and biosynthetic potential of basidiomycete polyketide synthases. Fungal Genet. Biol. 49:996-1003.
20. Lee, J.-K., Jung, H.-M., and Kim, S.-Y. 2003. 1,8-Dihydroxynaphthalene (DHN)-melanin biosynthesis inhibitors increase erythritol production in *Torula corallina*, and DHN-melanin inhibits erythrose reductase. App. Environ. Microb. 69:3427-3434.
21. Ludwig, N., Löhner, M., Hempel, M., Mathea, S., Schliebner, I., Menzel, M., Kiesow, A., Schaffrath, U., Deising, H., and Horbach, R. 2014. Melanin is not required for turgor generation but enhances cell wall rigidity in appressoria of the corn pathogen *Colletotrichum graminicola*. Mol. Plant-Microbe Interact. (in press).
22. McLean, R. J. 1979. Histological studies of resistance to soybean rust, *Phakopsora pachyrhizi* Syd. Aust. J. Agric. Res. 30:77-84.
23. Mendgen, K., Hahn, M., and Deising, H. 1996. Morphogenesis and mechanisms of penetration by plant pathogenic fungi. Ann. Rev. Phytopathol. 34:367-386.

24. Miles, M. R., Levy, C., Morel, W., Mueller, T., Steinlage, T., van Rij, N., Frederick, R. D., and Hartman, G. L. 2007. International fungicide efficacy trials for the management of soybean rust. *Plant Dis.* 91:1450-1458.
25. Miller, L. 2001. Microwave processing techniques for biological samples in a service laboratory. in: *Techniques and Protocols*, M. Giberson and R. Demaree, eds. Humana Press Inc., Totowa, NJ.
26. Money, N. P. 1989. Osmotic pressure of aqueous polyethylene glycols: Relationship between molecular weight and vapor pressure deficit. *Plant Physiol.* 91:766-769.
27. Money, N. P. 1997. Mechanism linking cellular pigmentation and pathogenicity in rice blast disease. *Fungal Genet. Biol.* 22:151-152.
28. Mueller, T. A., Miles, M. R., Morel, W., Marios, J. J., Wright, D. L., Kemerait, R. C., Levy, C., and Hartman, G. L. 2009. Effect of fungicide and timing of application on soybean rust severity and yield. *Plant Dis.* 93:243-248.
29. Posada-Buitrago, M. L., and Frederick, R. D. 2005. Expressed sequence tag analysis of the soybean rust pathogen *Phakopsora pachyrhizi*. *Fungal Genet. Biol.* 42:949-962.
30. Pryce-Jones, E., Carver, T., and Gurr, S. J. 1999. The roles of cellulase enzymes and mechanical force in host penetration by *Erysiphe graminis* f. sp. *hordei*. *Physiol. Mol. Plant Path.* 55:175-182.
31. Stone, C. L., McMahon, M. B., Fortis, L. L., Nuñez, A., Smythers, G. W., Luster, D. G., and Frederick, R. D. 2012. Gene expression and proteomic analysis of the formation of *Phakopsora pachyrhizi* appressoria. *BMC Genomics* 13:269-295.

32. Takano, Y., Kubo, Y., Shimizu, K., Mise, K., Okuno, T., and Furusawa, I. 1995. Structural analysis of *PKSI*, a polyketide synthase gene involved in melanin biosynthesis in *Colletotrichum lagenarium*. Molec. and Gen. Genet. 249:162-167.
33. Terhune, B. T., Bojko, R. J., and Hoch, H. C. 1993. Deformation of stomatal guard cell lips and microfabricated artificial topographies during appressorium formation by *Uromyces*. Exp. Mycol. 17:70-78.
34. Tremblay, A., Hosseini, P., Li, S., Alkharouf, N. W., and Mathews, B. 2013. Analysis of *Phakopsora pachyrhizi* transcript abundance in critical pathways at four time-points during infection of a susceptible soybean cultivar using deep sequencing. BMC Genomics 14:614 <http://www.biomedcentral.com/1471-2164/14/614>.
35. Tremblay, A., Li, S., Scheffler, B., and Mathews, B. 2009. Laser capture microdissection and expressed sequence tag analysis of uredinia formed by *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust. Physiol. Mol. Plant P. 73:163-174.
36. Tsai, H.-F., Wheeler, M., Chang, Y., and Kwon-Chung, K. 1999. A developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. J. of Bacteriol. 181:6469-6477.
37. Vittal, R., Paul, C., Hill, C. B., and Hartman, G. L. 2014. Characterization and quantification of fungal colonization of *Phakopsora pachyrhizi* in soybean genotypes. Phytopathology 104:86-94.

TABLES

Table 1. Documented appressorial turgor pressures in different fungal plant pathogens

Phylum	Species	Pressure	Melanin	Method	Reference
<i>Ascomycota</i>	<i>Magnaporthe oryzae</i>	8 MPa	Present	PEG8000 cytorhysis	(16)
	<i>M. oryzae</i>	5.8 MPa	Present	Glycerol cytorhysis	(8)
<i>Ascomycota</i>	<i>Colletotrichum kahawae</i>	2.6 MPa	Present	PEG8000 plasmolysis	(6)
<i>Ascomycota</i>	<i>C. graminicola</i>	5.35 MPa	Present	Waveguide deformation	(3)
	<i>C. graminicola</i>	3.9 MPa	Present	Mach-Zehnder-Microscopy and cytorrhysis	(21)
<i>Ascomycota</i>	<i>Erysiphe graminis</i> f.sp. <i>hordei</i>	2-4 MPa	ND ^a	PEG6000 plasmolysis	(30)
<i>Ascomycota</i>	<i>C. truncatum</i>	4-5 MPa	Present	PEG8000 plasmolysis	This study
<i>Basidiomycota</i>	<i>Uromyces spp.</i>	0.27-0.35 MPa	ND	ND	(30, 33)
<i>Basidiomycota</i>	<i>Phakopsora pachyrhizi</i>	5-6 MPa	Absent	PEG8000 plasmolysis	This study

^aNot described

Table 2. Appressorial turgor pressures (MPa) of *Phakopsora pachyrhizi* and *Colletotrichum truncatum* not treated or treated with melanin inhibitors from 6 to 48 hours post inoculation (hpi)

Treatment ^a	<i>P. pachyrhizi</i>				<i>C. truncatum</i>		
	6 hpi	12 hpi	24 hpi	48 hpi	16 hpi	24 hpi	48 hpi
Control	3.95 ± 0.38	6.05 ± 0.73	4.26 ± 0.32	2.73 ± 0.22	4.18 ± 0.05	4.58 ± 0.25	3.18 ± 0.40
Tricyclazole	4.05 ± 0.25	6.01 ± 0.93	4.23 ± 0.29	2.11 ± 0.54	3.56 ± 0.29 ^b	2.79 ± 0.15 ^b	2.87 ± 0.39
Niacin	4.14 ± 0.26	5.87 ± 0.56	4.13 ± 0.50	1.83 ± 0.42	4.29 ± 0.33	4.38 ± 0.22	3.95 ± 0.59

^a Spores were suspended in 0.1% Tween 20 aqueous solution. Tricyclazole and Niacin were also suspended in 0.1 % Tween 20 aqueous solution. One hundred spores were counted for each osmolality conditions for each time point, with a total of 500 spores measured to construct a regression line. Each value represents three biological replications.

^b Significantly different from control ($P < 0.05$).

Table 3. Appressorial turgor pressures (MPa) of *Phakospora pachyrhizi* in different concentrations of melanin inhibitors at 12 hours post inoculation

Concentration μM^a	Tricyclazole	Niacin
0	4.90 ± 0.24	
50	4.91 ± 0.43	4.88 ± 0.23
150	4.70 ± 0.24	4.53 ± 0.17
250	4.70 ± 0.28	4.66 ± 0.19

^a Spores were suspended in 0.1% Tween 20 aqueous solution as a control condition. Tricyclazole and Niacin were also suspended in 0.1 % Tween 20 aqueous solution. One hundred-fifty spores were counted for each osmolality conditions, with a total of 1200 spores measured to construct a regression line. Each value represents three biological replications.

FIGURES

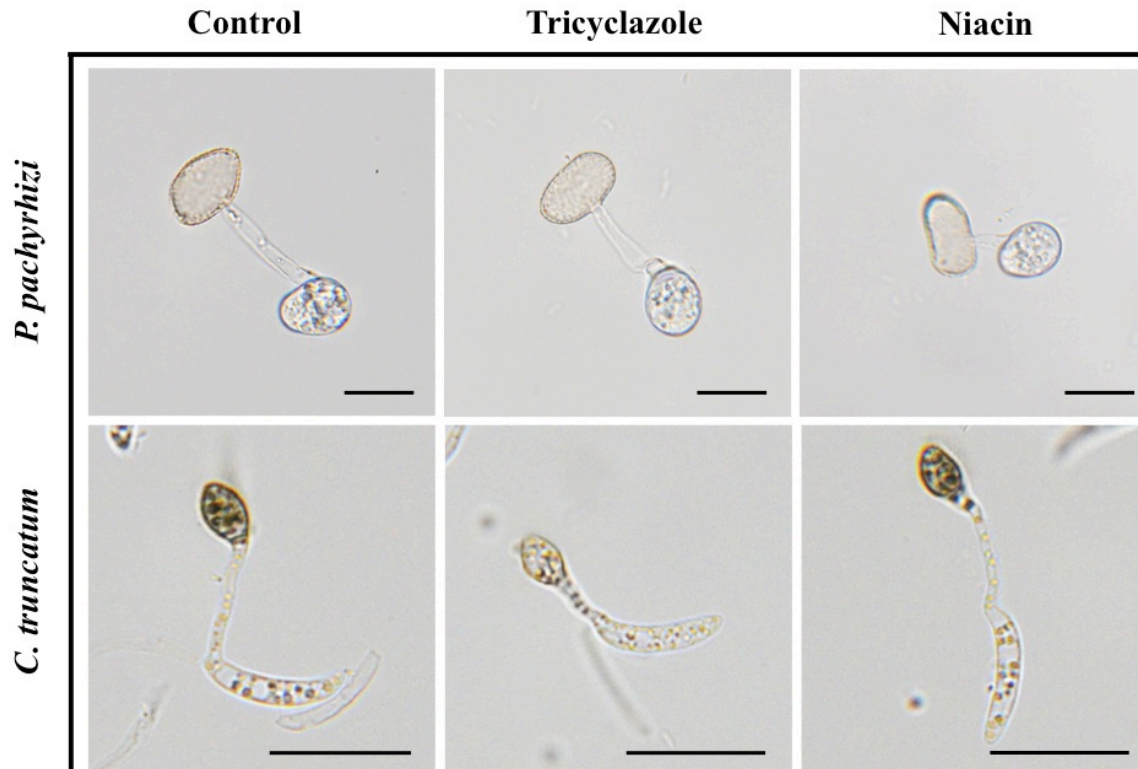


Fig. 1. Non-melanized appressoria of *Phakopsora pachyrhizi*. Germination of urediniospores and conidia of *P. pachyrhizi* and *Colletotrichum truncatum*, respectively, to produce appressoria. Both melanin inhibitors had no impact on *P. pachyrhizi* appressoria. In contrast, the melanized appressorium of *C. truncatum* became light reddish and more transparent in the presence of DHN-melanin inhibitor tricyclazole. The bar represents 25 μm for all pictures.

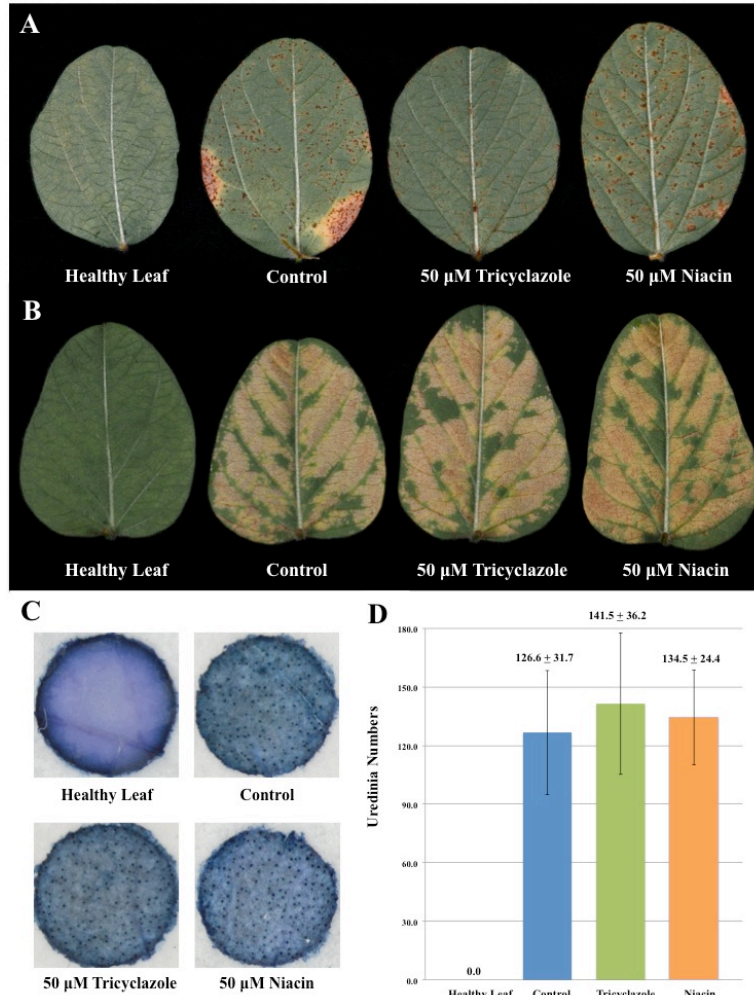


Fig. 2. Pathogenicity of *Phakopsora pachyrhizi* and *Colletotrichum truncatum* in the presence of different melanin inhibitors. **(A)** Necrosis caused by the soybean anthracnose pathogen, *C. truncatum*. The necrotic area was reduced when treated with 50 μ M tricyclazole. **(B)** Symptoms of soybean rust disease were not reduced by treatment of tricyclazole or niacin. **(C)** Uredinia were clearly visualized after staining with 0.1 % lactophenol cotton blue. The leaf disk diameter was 5 mm. **(D)** Quantification of uredinia revealed no significant difference between treatments. Six leaf disks were measured for each treatment with three biological replications.

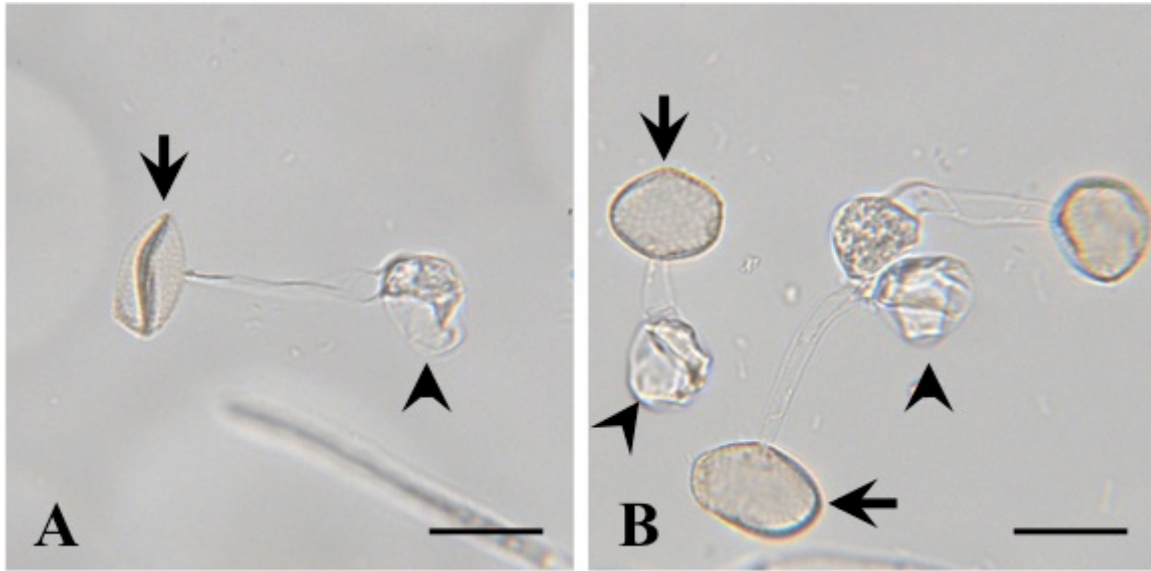


Fig. 3. The cell wall permeability of urediniospores and appressoria of *Phakopsora pachyrhizi*. **(A)** Appressoria treated with 6 MPa PEG 8000 solution. **(B)** Appressoria treated with 5.8 MPa glycerol solution. Arrowheads indicate the empty site of plasmolyzed appressoria. Arrows indicate the empty urediniospores. In both treatments, the cell walls of appressoria retained their original spherical shape but the cytosols shrank in the site opposite the arrowhead. However, PEG 8000 caused cytorrhysis on empty urediniospore. The difference in permeability of the empty urediniospore indicated that fungal cell wall selection of molecule size occurred on the cell walls of empty urediniospores but not the cell walls of appressoria. The bars represent 25 μm .

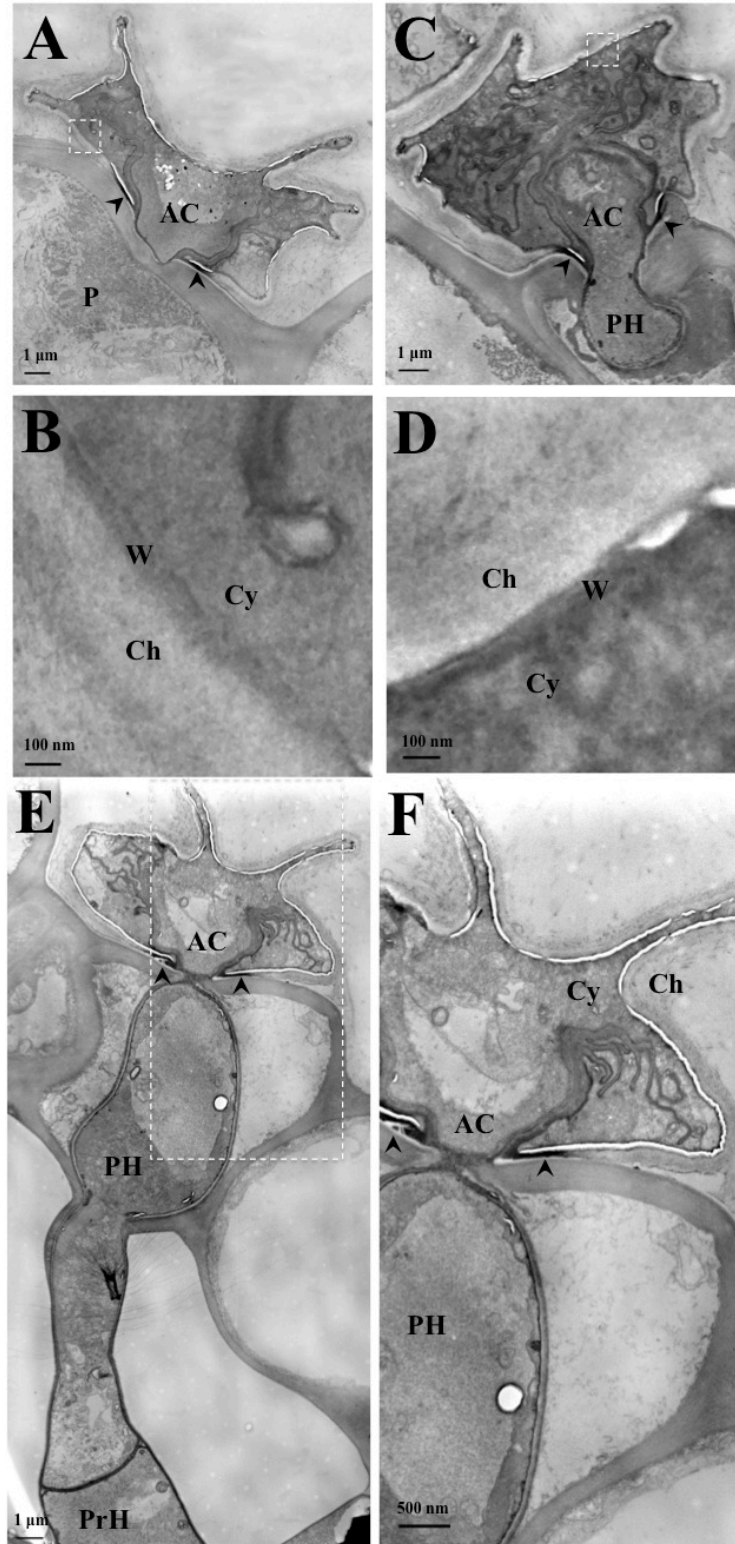


Fig. 4. TEM analysis of material between appressorial cell walls and cytoplasmic membrane of *Phakopsora pachyrhizi*.

Fig. 4. Legend. AC: appressorial cone; Ch: chitin; Cy: cytosol; P: papillae; PH: penetration hyphae; PrH: primary hyphae; W: waxy layer; Arrowhead indicates dark materials. **(A)** The early stage of penetration. Papillae formed to prevent the invasion of *P. pachyrhizi* penetration hyphae. Appressorial cone started to form at this stage. **(B)** The amplified image of the white dash block in A. No dark material could be found in between chitin cell wall and cytosol. However, a grey waxy layer presented in between. **(C)** Penetration hyphae developed and invaded into epidermis. Dark material became more obvious only around the penetration site and did not surround the appressorium or was not observed between the appressorial cell wall and cytosol. **(D)** The amplified image of the white dash block in C. Grey waxy layer became more obvious. **(E)** The penetration hyphae invaded through the epidermis, and developed into primary hyphae for further colonization. **(F)** The enlarged image of the white dash block in E. Dark material was limited at penetration site but not other parts between the cell wall and cytosol of the appressorium.

APPENDIX

Table A1. The translation of PEG 8000 between molar and osmotic pressure

C (molar)	Mpa ^a	Preparation (per 50 ml)
0.0000	0.000	0.00 g
0.0376	2.000	15.04 g
0.0500	4.001	20.00 g
0.0596	6.008	23.84 g
0.0677	8.000	27.08 g

^a Translation equation: $\Pi = \alpha C + \beta C^2$. $\alpha = -28.16$ and $\beta = 2163.84$ for PEG8000. The conversion of PEG concentration to osmotic pressure Π (MPa) is given by the equation: $\Pi = \alpha C + \beta C^2$, while Π is the osmotic pressure (MPa), C is the molar concentration (M) of PEG and α and β are constants from different molecular weights of PEGs.

Table A2. Development of *P. pachyrrhizi* in the presence of melanin inhibitors

hpi	Germination (%)			Appressoria Formation (%)			Primary Hyphae Formation (%)		
	Control ^a	Tricyclazole ^a	Niacin ^a	Control	Tricyclazole	Niacin	Control	Tricyclazole	Niacin
6	51.6 ± 7.1	68.0 ± 12.4	65.1 ± 7.1	57.6 ± 6.7	50.7 ± 9.1	49.9 ± 7.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
12	53.1 ± 7.3	53.3 ± 6.6	62.9 ± 11.1	59.1 ± 9.9	58.0 ± 8.9	52.5 ± 9.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
24	71.1 ± 11.4	67.8 ± 12.8	70.4 ± 11.3	81.7 ± 8.9	75.8 ± 4.6	66.9 ± 7.4	2.9 ± 3.9	1.0 ± 2.9	1.5 ± 2.3
48	87.3 ± 3.3	91.8 ± 4.7	92.9 ± 3.9	80.3 ± 7.0	87.0 ± 3.7	90.7 ± 4.4	22.7 ± 6.3	21.7 ± 6.5	25.5 ± 8.6

^a Spores were suspended in 0.1 % Tween 20 solution.

Table A3. Development of *C. truncatum* in the presence of melanin inhibitors

hpi	Germination (%)			Appressoria Formation (%)			Melanized Appresoria (%)		
	Control ^a	Tricyclazole ^a	Niacin ^a	Control	Tricyclazole	Niacin	Control	Tricyclazole	Niacin
16	83.1 ± 6.6	73.3 ± 5.8	75.3 ± 7.1	29.5 ± 6.7	38.7 ± 5.8	30.1 ± 11.0	27.5 ± 11.5	2.0 ± 4.1	42.5 ± 8.6
24	92.9 ± 6.1	78.7 ± 5.5	87.3 ± 6.5	73.7 ± 8.6	53.5 ± 10.2	63.8 ± 6.9	83.7 ± 3.7	0.6 ± 1.7	86.5 ± 6.8
48	94.2 ± 4.6	94.2 ± 3.2	95.1 ± 3.2	70.8 ± 13.8	73.2 ± 14.6	81.2 ± 8.7	93.7 ± 3.5	1.6 ± 2.0	83.8 ± 10.5

^a Spores were suspended in 0.1 % Tween 20 solution.